LOSt and found across the mycobacterial cell envelope

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General Discussion

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The bacterial cell envelope is a vital boundary to separate the cytosol from the outside world and offers the first line of defence against antibacterial agents. Due to its high lipid content and structural organization, the mycobacterial cell envelope presents an exceptionally efficient permeability barrier for hydrophilic molecules and antibiotics. On the other hand, this barrier poses a challenge for the bacterium in the uptake of nutrients and export of proteinaceous virulence factors. Porins are necessary for the transport of small hydrophilic solutes, but not much is known about the channel-forming proteins residing in the unusual mycolate outer membrane [21]. In recent years, type VII secretion (T7S) systems, which are key to mycobacterial pathogenesis, are beginning to be unravelled. Different T7S systems play an essential role in specific steps of the mycobacterial infection route. Whereas the ESX-1 system is involved in early steps of infection, the ESX-5 system has a role at a later stage. The ESX-3 system is important for bacterial viability and metabolism. Based on homology predictions and genetic studies, these three ESX systems are thought to have a similar working mechanism, although our structural and mechanistic knowledge of T7S is fairly limited. The more detailed understanding of the specialized Type I to VI secretion (T1S-T6S) systems, which are found in Gram-negative bacteria, could help to interpret our findings for T7S (see below). Pathogenic mycobacteria also rely on the accessory Sec pathway for their infectivity, but the involved effector proteins are unknown. This thesis was specifically dedicated to studies of the T7S pathway ESX-5, but also investigated other aspects of mycobacterial protein secretion and the cell envelope, which are summarized and discussed below.

Chaperones that assist in type VII secretion

PE and PPE proteins, which represent roughly 10% of the coding capacity of *Mycobacterium tuberculosis* and *Mycobacterium marinum*, are a major substrate family for the T7S pathway and are thought to dimerize before translocation. Many PE and PPEs are secreted via ESX-5, but some are directed to the ESX-1 pathway. The general T7S secretion motif YxxxD/E, which is present on the PE partner and is essential for the secretion of both PE and PPE proteins, does not explain how these proteins are specifically targeted to their cognate secretion systems [11]. In chapter 5, we have investigated the role of $EspG_5$, a cytosolic component of the ESX-5 pathway, and found that it specifically interacts with cognate PE and PPEs. The fact that $EspG_5$ does not bind PE and PPE proteins that are directed to the ESX-1 system, but its paralogue of the ESX-1 pathway, *i.e.* $EspG_1$, does, clearly indicates that EspG proteins have a system-specific role in T7S. Our analysis also showed that EspG proteins assist specifically in PE/PPE secretion and do not interact with the classical T7S substrate family of small Esx proteins. The link between PE/PPE and EspG families seems to be conserved in another mycolata species, *Nocardia farcinica*, since genome sequencing has shown that the respective gene homologues are in the same putative operon. In accordance, *Mycobacterium leprae*, which has a minimal genome that has undergone extensive gene loss during evolution, has lost *pe35* encoded by the ESX-1 system and also $espG_1$ is only present as a pseudogene. Interestingly, the ESX-5 cluster of *M. leprae* has retained $espG_5$, but lost both *esxMN* and most *pe/ppe* genes. Because $EspG_5$ is still produced, this indicates that possibly some of the remaining PE/PPE proteins of

M. leprae [15] are binding to this protein and concomitantly transported via ESX-5.

T7S substrates seem to depend on multiple signals to ensure their specific targeting. A similar mechanism has been described for the T3S pathway, which is used by Gram-negative bacteria to directly inject proteins into host cells via the so-called injectisome. T3S effector proteins contain an N-terminal secretion signal, but also depend on several classes of cytosolic chaperones. Many of the T3S chaperones are highly substrate-specific and fulfil their task independent of ATP consumption [27]. In complex with their substrate, these chaperones provide a three-dimensional secretion signal and greatly enhance translocation efficiency [18]. It seems likely that EspG proteins act in a similar way as targeting factors. Although the functional relevance of the interaction between EspG proteins and their cognate PE/PPE complex is still unknown, the stability of the interaction is also striking. Interestingly, no interaction between $EspG_5$ and PE/PPEs was detected in a strain with a functional ESX-5 system, probably due to the fact that the association is transient and disrupted prior to translocation. We have shown that $EspG_5$ does not depend on the general T7S motif YxxxD/E (chapter 5) and this motif is thus likely exposed to interact with other components, perhaps the cytosolic domain of EccC. $EccC_1$ was previously shown to interact with the C-terminal tail of EsxB, which also contains the YxxxD/E motif [8]. Future experiments should determine whether the EspG/PE/PPE complex interacts with one of the other type VII components.

Another characteristic feature of the T3S pathway is that, although the sequence similarity of its independent chaperones is low, within the specific classes their structure is highly conserved [18]. Sequence similarity between $EspG_5$ and the other EspG proteins is also notably low, and was initially a reason not to name this protein $EspG_5$ [4]. This dissimilarity might be due to the fact that $EspG_5$ serves a larger group of PE/PPE proteins than the other EspG proteins, since ESX-5 is the major secretory pathway for PE/PPEs. ESX-2- or ESX-3-dependent secretion of PE/PPEs remains to be shown, but is not unlikely because *pe/ppe* genes are also present in these genomic regions. Additionally, similar to the T3S chaperones, the folding of EspG proteins could be highly conserved. Size exclusion chromatography analysis of the $EspG_5$-PPE41/PE25 complex in chapter 5 has indicated that $EspG_5$ has a globular structure. Elucidation of the structure of the EspG proteins in complex with the PE/PPEs will be required to provide more insight in this issue.

Surface release of T7-secreted proteins

$EspG_5$ was discovered to be essential for ESX-5-dependent secretion in a screen for transposon mutants deficient in PPE41 secretion. Later it was shown that the ESX-5 system is also the major translocation pathway for PE_PGRS proteins [1, 16]. This extensive protein family is characterized by a C-terminal domain that contains multiple tandem repeats of glycine and alanine, which can be recognized by a single antiserum [1]. In chapter 2, we performed a transposon mutant screen, using the anti-PE_PGRS serum as readout for active ESX-5-dependent secretion, in order to identify novel essential components of ESX-5. Instead, we found a large number of mutants involved in the biosynthesis of the specific glycolipid lipooligosaccharide (LOS). The mycobacterial cell wall contains a large number of different lipids. However, since no genes involved in biosynthesis of other glycolipids were found to be disrupted in our screen, the link between LOS biosynthesis and PE_PGRS proteins seems to be specific.

Further investigation showed that disruption of the LOS biosynthesis pathway does not affect translocation,

but results in increased attachment of PE_PGRS proteins to the cell wall. This effect was not limited to the PE_PGRS proteins as the major capsular protein EspE was similarly affected. Although PE_PGRS proteins are described to be mainly connected to the cell envelope [12], it is not known how. Both the PGRS domain and a region adjacent to the PE domain have been proposed to serve as an anchor for the cell wall [12], but what they attach to is not known. Our results indicate that in *M. marinum,* PE_PGRS proteins localize partially to the loosely associated capsule structure and their release is augmented in the presence of LOS(-IV). To examine whether we could cross-complement this phenotype we also studied the effect of co-culturing LOS mutant bacteria with a LOS-producing ESX-5 mutant strain. However, these mixed cultures did not reverse the defect. This preliminary experiment suggests that LOS does not function as a detergent-like molecule. On the other hand, the externally added LOS might not affect the capsular layer in the same way as the homologous biogenesis of LOS molecules.

Notably, most species of the *M. tuberculosis* complex do not produce LOS and usually have a rough colony morphology. Similar colony phenotype was also observed for LOS mutants in *M. marinum.* In accordance, we detect significantly less PE_PGRS proteins in the culture filtrate of *M. tuberculosis* than of *M. marinum*. Also, when a transposon screen for PE_PGRS secretion was performed using *M. bovis* BCG, we could not detect sufficient amount of secreted PE_PGRS to perform a successful screen. Smooth colony morphology of the *M. tuberculosis complex* is uncommon, but has been described for *M. canettii*, a strain that also produces LOS. It might be interesting to test whether the release of PE_PGRS proteins for this strain is affected. Preliminary experiments (Aniek van der Woude in collaboration with Roland Brosch) showed no clear differences in the culture filtrate. However, LOS is a species-specific glycolipid and it is not known whether the structure of LOS from *M. marinum* is the same as *M. canettii*. Curiously, a large part of the LOS biosynthesis locus is preserved in *M. tuberculosis.* Possibly, this region is involved in the production of another glycolipid, but thus far the function of these genes in this species is unknown.

The unbiased genetic approach taken in chapter 2 to identify (unexpected) components essential for ESX-5-dependent secretion merely identified components involved in surface release. As no ESX-5 genes were found in this screen, these data also suggest that the structural ESX-5 components are essential for viability in *M. marinum*. This seems in contrast to *M. tuberculosis,* in which most ESX-5 genes can be disrupted [6, 17]. However, the situation in *M. tuberculosis* might also be more complex than anticipated, because the two different strains of *M. tuberculosis* (H37Rv and CDC1551) that have been studied in most detail do show differences in essentiality of ESX-5 genes. Whereas high-throughput transposon insertion site sequencing in H37Rv suggests that at least the structural genes of ESX-5 are essential, a similar approach in CDC1551 generated hits in most ESX-5 genes. How do we explain essentiality of ESX-5 in *M. marinum*? One possibility is that one or more substrates secreted via ESX-5 are crucial for viability. However, since many mycobacteria do not contain ESX-5 this is perhaps not the most probable explanation. Another possibility is that (some) ESX-5 substrates accumulate in the cytosol and thereby disrupt some vital feature, such as the integrity of the cytoplasmic membrane. In this respect, it is interesting to note that the number of PE and PPE proteins encoded by the *M. marinum* genome is much more extensive than in *M. tuberculosis* [26] and some of

these additional members could have different properties. Alternatively, a new yet unidentified ESX-5 substrate, perhaps with the YxxxD/E motif [11], might be essential in *M. marinum.*

The outer membrane component of the T7S pathway

The T7S pathway clearly orchestrates secretion of its effector proteins across both the inner (IM) and the outer membrane (OM). Convincing experimental evidence showing whether this is a one- or a two-step process is still lacking. Of note, T7S structural components are all predicted to associate with the IM [4] and how secretion across the OM is accomplished is currently unknown. One possibility is that a novel unrelated protein (complex) forms the outer membrane channel. No such component could be identified in the genetic screen of chapter 2. Alternatively, one of the components encoded by the ESX cluster is the missing OM protein.

For the ESX-5 cluster, it has been shown that four conserved components, $EccB_5$, $EccC_5$, $EccD_5$ and $EccE_5$, form a large membrane complex [17]. The EccC and EccD components are obvious IM components: EccD contains up to twelve predicted transmembrane domains, whereas EccC only has two transmembrane domains, but has a large cytoplasmic domain that contains three putative ATP binding sites [4]. EccC belongs to the FtsK/SpoIIIE-like protein family and is homologous to the VirD4 component of the T4S system [10]. This leaves EccB and EccE as putative OM proteins. If the identified membrane complex indeed spans both the IM and the OM, EccE would be the prime candidate for the OM component; limited proteolysis showed that the predicted transmembrane domains of $EccE_5$ were sensitive to proteolytic degradation even when embedded in the cell envelope [17]. Furthermore, EccE is a relatively recently evolved T7S component: it is lacking in the ancient ESX-4 system and in T7S systems of species without an outer membrane [14].

It is also possible that mycosin MycP is the outer membrane component. This serine protease of the subtilisin family associates with the cell envelope, but not with the core ESX-5 secretion machinery [17]. The differential detergent extraction of mycobacterial cell envelopes and subsequent nanoLC-MS/MS analysis described in chapter 3, showed that $MycP_1$ and $MycP_3$ were highly detergent extractable, which suggests that they could localize to the OM. $MycP_1$ has been indicated to specifically cleave the ESX-1 substrate EspB and since no cleaved EspB is found in the cellular fraction this process likely occurs in the periplasm or at the surface [22]. Interestingly, although MycP is essential for the T7S pathway, its protease activity is not [22] and E.N.G. Houben, unpublished observations], indicating that this protein could also have a structural role in the T7S machinery. If one of the core components of T7S indeed forms the OM channel, the organization could also be similar to T4S. Here, multiple copies of the inner membrane protein VirB10 span the periplasm and form an unusual α-helical barrel in the OM [9].

Alternatively, T7S could be organized similar to the T6S pathway, in which the interdependent secreted proteins Hcp and VgrG are proposed to form tubules that puncture the OM and could assist in secretion of other effector proteins [5]. Many TS7 substrates, such as the ESX-1 substrates EsxA/B and EspAC and EspB, are also mutually dependent for secretion, but the reason for this interdependence is unknown. Perhaps, the EsxA/B-like proteins, which are conserved in every ESX system, could have a structural role and function as OM channel. The fact that EsxA has been associated with membrane rupture also supports this model. Another option is that specific PE and PPE proteins act as the outer membrane channel in

T7S. In bioinformatic studies for ◎ barrel folding, specific PE and PPE proteins have been proposed to form outer membrane channels [23, 25]. In addition, disruption of some PE and PPE proteins clearly affected the cognate T7S pathway [6] and unpublished observations], which could indicate a structural role for these enigmatic proteins.

Other proteins residing in the mycolate outer membrane

One of the reasons why we have not identified the OM channel of T7S, is that the identification of mycobacterial outer membrane proteins (MOMPs) in general already presents a major challenge in the field [21]. Although the permeability of the mycobacterial cell envelope for hydrophilic solutes is much lower than observed for Gram-negative diderm bacteria, porins should be present to sustain growth. Even though they remain largely unidentified, these MOMPs are expected to be essential for nutrient uptake and (antibiotic) efflux processes. MspA, the major porin of *M. smegmatis,* seems to function as a main diffusion pore for glucose, phosphate, but also metal ions. MspA forms an octameric goblet-like β-barrel and is a representative of a novel class of outer membrane proteins of which numerous homologues are present in fast-growing mycobacteria and other mycolates [20]. However, no homologues of this protein have been identified in the slow-growing *M. tuberculosis*. The two proteins that have been described to reside in the OM of *M. tuberculosis, i.e.* OmpATb and MctB, were used as OM markers in the assay that we developed to identify novel MOMPs in chapter 3. We found that we could specifically differentially solubilize these proteins from isolated cell envelopes using the non-ionic detergent Octyl-β-glucoside, while typical IM proteins, like FtsH and $EccC_5$, remained in the pellet fraction. Proteins that were similarly extracted thus presented interesting novel candidate MOMPs.

OmpATb and MctB, which have a role in ammonium burst and copper efflux, respectively, are likely not classical porins of β-barrel conformation. β-barrel structures contain a variable but even amount of β-strands and are common for most porins and other proteins present in the outer membrane of Gram-negative bacteria. These classical β-barrels are generally transported across the IM by the Sec machinery and subsequently inserted into the OM by a complex known as the β-barrel assembly machinery (Bam) complex. No homologues of the Bam complex have been identified in mycobacteria, indicating that if insertion of OM proteins is organized similarly a different mechanism is involved. Recent structural work has revealed that outer membrane localization in Gram negative bacteria is not restricted to proteins containing a β-barrel. An example was previously mentioned, the VirB10 protein of T4S. Another α-helical protein in the OM is Wza, which is involved in transport of capsular polysaccharides to the cell surface of *E. coli.* Wza folds into four domains that adopt an octameric channel. While the N-terminal regions span the periplasm, the C-terminal amphipathic α-helix inserts into the OM [13]. Perhaps, similar α-helical barrel conformations will be identified for mycobacteria and related species. For example, the PorB protein of the mycolic-acid producing *Corynebacterium glutamicum* was proposed to have an oligomeric α-helical structure [28].

The structure of the channel-forming protein that we identified in chapter 3, MMAR_0617, is unknown. Bioinformatics analysis predicts that the protein contains several α-helices, but also β-strands. Importantly, the C terminus of MMAR_0617 was shown to be crucial for its membrane localization, as C-terminal truncation resulted in a soluble protein.

This could suggest that the N-terminal region of MMAR_0617 presents a large soluble periplasmic domain with the C terminus inserting into the OM, similar to Wza. However, the structure of this threonine-rich region is difficult to predict, since it is low in complexity and shows little homology to known proteins. In addition, the C terminus is subject to numerous posttranslational modifications, such as glycosylation. These glycosylated residues will not insert into the membrane, but might instead be exposed to the surface to interact with the host immune system. Another interesting observation is that overexpression of this protein does not significantly affect bacterial viability or antibiotic sensitivity (not shown). In that respect, it seems likely that the channel formed by MMAR_0617 is gated. It will be interesting to obtain a *mmar_0617* mutant to gain insight into the function of this novel type of porin and its role in mycobacterial virulence.

The accessory Sec pathway

Not only T7S, but also the accessory Sec pathway was studied in this thesis. Both translocation systems have a distinct role in mycobacterial virulence and they are very different. Whereas each T7S system is encoded by a large operon, the accessory Sec system is thus far centred around a single gene, *secA2*. Disruption of *secA2* affects pathogenesis of *M. marinum* (chapter 4) and *M. tuberculosis*. An *M. tuberculosis secA2* mutant shows enhanced apoptosis of infected macrophages, whereas wild-type bacteria interfere with apoptosis and induce a more necrotic-type of cell death. Apoptosis of host cells has been implicated in protective immunity for tuberculosis and the *secA2* mutant is therefore also a promising candidate for vaccine studies, especially in an HIV-infected population [19]. The basis for this attenuation is largely unknown, but our results from chapter 4 strongly suggest that the SecA2-dependence of PknG for its membrane localization strongly contributes to the observed phenotype.

SecA proteins generally have a dual function: the ATPase domain provides energy for translocation, while a substrate binding domain can function as a chaperone for translocation. Translocation via the accessory Sec pathway has been proposed to be funnelled into the canonical Sec translocon and also depends on SecA1. This hypothesis is supported by the fact that depletion of SecA1 affects SecA2-dependent translocation [24] and that many substrates of the accessory Sec pathway are not completely SecA2-dependent (chapter 4). There are currently no indications if and how substrates of the Sec pathway are transported across the mycolate outer membrane. The available evidence indicates that certain proteins with signal sequences are indeed specifically secreted to the cell surface or into the medium, implying the need for a special mycolate outer membrane transport system.

Substrates of the Sec pathway are generally characterized by an N-terminal signal sequence, which is cleaved upon translocation. This signal sequence serves as a targeting motif, which is recognized by SecA. How specificity for either SecA1 or SecA2 is accomplished is not known. For a substrate of the accessory Sec pathway of *Streptococcus gordonii*, a specific recognition domain has been identified adjacent to the N-terminal signal sequence. This domain, which seems to form an amphipathic helix, was shown to specifically interact with SecA2 [2, 3]. No such domain has yet been determined for mycobacterial SecA2 substrates. Secretion is probably also organized differently for the SecA2-only systems, which serve multiple substrates, in contrast to the more specific SecA2-SecY2 system of *S. gordonii*. However, it does seem likely that SecA2 substrates share a common recognition domain. The

identification of multiple novel substrates containing a signal sequence, such as DppA and MMAR_3060 in chapter 4, will contribute to elucidation of such a domain, especially because homologues of these mycobacterial SecA2 substrates are also dependent on SecA2 in the distantly related Gram-positive species *Listeria monocytogenes*. Interestingly, also a number of SecA2 substrates without putative signal sequences, such as SodA [7] and PknG (chapter 4), have been identified. Whether the dependence of these proteins on the accessory Sec pathway is direct or indirect still remains to be shown. Dedicated interaction studies should be performed to show the basis of the interaction between SecA2 and its substrates. These studies might also identify a domain, which determines specificity for SecA2.

Concluding remarks

When the tubercle bacillus was discovered as the causative agent of the consumption by Robert Koch in 1882, it was generally assumed that tuberculosis would soon be eradicated. After 130 years, although we have gained considerable insight in the mechanisms of pathogenesis of *M. tuberculosis,* we are still failing to fulfil Koch's prediction. However, Koch at the time probably also did not realize what an extraordinary prokaryote this tuberculosis bacillus is. Recently, the knowledge on the mycobacterial cell envelope and its dedicated protein secretion systems has significantly expanded. This thesis also has given insight in the broad variety of transport processes across the mycobacterial cell envelope. While we initially got LOSt in broad-scope studies for the T7S system ESX-5, these studies led to interesting and unexpected findings about other processes in the cell envelope. Continued efforts to characterize the mycobacterial cell envelope and secreted proteins will allow us to devise new approaches to fight this persistent disease.

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